

**Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas.**

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Vascular endothelial growth factor (VEGF) is the major endothelial mitogen in central nervous system neoplasms and it is expressed in 64-95% of glioblastomas (GBMs). Tumour cells are the main source of VEGF in GBMs whereas VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells. Infiltrating tumour cells and newly-formed capillaries progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). Recent studies have shown that VEGF expression and bioavailability can be modulated by MMPs. We reported previously that the expression of MT1-MMP in human breast cancer cells was associated with an enhanced VEGF expression. We used quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expression of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels; activated forms of MMP-2 and MMP-9 were present in 8/18 and 7/18 of GBMs. A majority of GBMs (17/20) also expressed high levels of VEGF, as previously reported, with strong correlation between VEGF and MT1-MMP gene expression levels, and double immunostaining showed that VEGF and MT1-MMP peptides co-localize in tumour and endothelial cells. Our results suggest that the interplay between metalloproteinases and VEGF previously described in experimental tumours may also be operative in human GBMs. Because of its dual ability to activate MMP-2 and to up-regulate VEGF, MT1-MMP might be of central importance in the growth of GBMs and represent an interesting target for anti-cancer treatments. Copyright 2003 Wiley-Liss, Inc.

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## VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION CORRELATES WITH MATRIX METALLOPROTEINASES MT1-MMP, MMP-2 AND MMP-9 IN HUMAN GLIOBLASTOMAS

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Vascular endothelial growth factor (VEGF) is the major endothelial mitogen in central nervous system neoplasms and it is expressed in 64–95% of glioblastomas (GBMs). Tumour cells are the main source of VEGF in GBMs whereas VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells. Infiltrating tumour cells and newly-formed capillaries progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). Recent studies have shown that VEGF expression and bioavailability can be modulated by MMPs. We reported previously that the expression of MT1-MMP in human breast cancer cells was associated with an enhanced VEGF expression. We used quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expression of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels; activated forms of MMP-2 and MMP-9 were present in 8/18 and 7/18 of GBMs. A majority of GBMs (17/20) also expressed high levels of VEGF, as previously reported, with strong correlation between VEGF and MT1-MMP gene expression levels, and double immunostaining showed that VEGF and MT1-MMP peptides co-localize in tumour and endothelial cells. Our results suggest that the interplay between metalloproteinases and VEGF previously described in experimental tumours may also be operative in human GBMs. Because of its dual ability to activate MMP-2 and to up-regulate VEGF, MT1-MMP might be of central importance in the growth of GBMs and represent an interesting target for anti-cancer treatments.

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**Key words:** VEGF; MMPs; glioblastomas; brain; tumor

Angiogenesis is critical for the development of normal tissue and solid tumours. This process includes the degradation of the extracellular matrix (ECM) and the proliferation, migration and differentiation of endothelial cells, and is finely regulated by inhibitory and promoting factors.<sup>1,2</sup> Among positive factors, vascular endothelial growth factor (VEGF) has been proposed as the major endothelial mitogen in central nervous system (CNS) neoplasms.<sup>3</sup> Strong VEGF expression has been detected by immunohistochemistry in 64–95% of glioblastomas (GBMs).<sup>4–6</sup> GBMs are the most common malignant primitive tumours of the CNS in adults.<sup>7</sup> Microvascular proliferation is characteristic of these tumours and is an essential WHO diagnostic criteria.<sup>7</sup> Tumour cells are the main source of VEGF in GBMs whereas VEGF receptors are predominantly expressed by endothelial cells.<sup>8,9</sup> These receptors differ both in terms of affinity and transduction signaling.<sup>10–12</sup> VEGFR-1 (flt-1) and VEGFR-2 (flk/KDR) belong to the Class II tyrosine-kinase receptor family. VEGFR-1 has a soluble isoform (sVEGFR-1) that modulates VEGF availability.<sup>8,13</sup> Neuropilin-1 (NRP1) is a co-receptor for VEGF that increases by 10-fold the affinity of the VEGF<sub>165</sub> isoform for VEGFR-2.<sup>14</sup> NRP1 is thought to modulate VEGF-mediated tumour angiogenesis in human malignant astrocytomas.<sup>10</sup> The coordinated up-regulation of VEGF and its receptors appears as a critical event in the control of angiogenesis.<sup>8,12</sup>

Infiltrating tumour cells and newly-formed capillaries progress through the ECM by local proteolysis involving matrix metalloproteinases (MMPs).<sup>15,16</sup> MMPs are proteolytic enzymes that are synthesized as inactive zymogens. Their activation requires the removal of a propeptide by proteinase cleavage and can be inhibited by various tissue inhibitors of MMPs (TIMPs). Most MMPs are secreted as soluble enzymes but a subset of them are inserted in the cell membrane by a transmembrane domain or by a glycosylphosphatidyl-inositol anchor and are classified as membrane-type MMPs (MT-MMPs).<sup>17,18</sup> In cultured tumour cells, MT-MMPs tend to accumulate on the cytoplasmic membrane of invadopodia where they selectively mediate local pericellular proteolysis. GBMs express high levels of MT1-MMP, MMP-2 and MMP-9.<sup>19,20</sup> Among these MMPs, MT1-MMP might play a central role in the remodeling of the ECM as this membrane-bound protease is able to activate MMP-2 and MMP-13.<sup>21,22</sup> Moreover, MT1-MMP has been shown to promote cell migration in various carcinoma cell lines by its ability to cleave laminin-5, a major constituent of basement membrane,<sup>23,24</sup> and through the processing of CD44H (the major receptor for hyaluronan)<sup>25</sup> and of  $\alpha_5\beta_3$  integrin.<sup>26</sup>

MT1-MMP is involved in both developmental and tumour angiogenesis.<sup>27</sup> MT1-MMP overexpression in human melanoma cells has been associated with enhanced *in vitro* invasion and increased *in vivo* tumour growth and vascularization.<sup>28</sup> We have shown previously that in MCF-7 breast cancer cells, VEGF transcription is upregulated when MT1-MMP is overexpressed.<sup>29</sup> Up-regulation of VEGF by MT1-MMP has also been reported in a model of human glioma xenograft by Deryugina *et al.*<sup>30</sup> These experimental data suggest a link between MT1-MMP and the VEGF network. We have tested for the presence of such a link in human glioblastomas. We compared the expression of VEGF and its receptors (VEGFR-1, sVEGFR-1, VEGFR-2, NRP1) with

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MT1-MMP, MMP-2, MMP-9 and TIMP-2 in a series of 20 GBMs and 5 normal brains. Using quantitative RT-PCR, gelatin zymography, Western blot and immunohistochemistry, we showed a strong correlation between the expression of VEGF, MT1-MMP, MMP-2 and MMP-9 in GBMs. These results are in accordance with previous *in vitro* studies and add to the evidence of an interplay between VEGF and MMPs in the progression of human GBMs.

#### MATERIAL AND METHODS

##### Patients

We studied 20 GBMs diagnosed at the Laboratory of Neuropathology-CHU Liège between 1997 and 2001. The series included 17 primary GBMs (*i.e.*, no previous history of lower grade diffuse astrocytoma) and 3 secondary GBMs (*i.e.*, previous history of lower grade diffuse astrocytoma). Clinical information on these 20 cases have been reported previously as part of a larger series.<sup>31</sup> The gender ratio was 1/1, and the age at time of diagnosis ranged from 41–79 years (mean 56 years). Normal brain cortex and white matter were obtained from 5 patients with intractable epilepsy treated by partial temporal lobectomy. Histological examination of these specimens showed severe hippocampal sclerosis; frozen tissue was sampled from microscopically normal inferior temporal gyri. Our study was approved by the Ethical Committee of the Faculty of Medicine of the University of Liège.

##### RNA extraction and cDNA synthesis

Total RNA was extracted from cryosections with RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed with a ThermoScript reverse transcriptase (ThermoScript RT-PCR System, Invitrogen, Carlsbad, CA) and random hexamers as primers.

##### Primers

Primers pairs used in our study are described in Table I. Primers for the VEGF gene were chosen to distinguish between VEGF<sub>189</sub>, VEGF<sub>165</sub>, VEGF<sub>145</sub> and VEGF<sub>121</sub> mRNA isoforms. Intron-spanning primers and probes for the TaqMan system (primers for VEGFR-1 (Flt-1), sVEGFR-1, VEGFR-2 (KDR/Flk-1) and NRPI) were designed to meet specific criteria by using Primer Express software (Perkin Elmer, Foster City, CA). All primers were synthesized by Eurogentec (Liège, Belgium). The 5'- and 3'-end nucleotides of the probe were labeled with a reporter (FAM = 6-carboxy-fluorescein) and a quencher dye (TAMRA = 6-carboxy-tetramethylrhodamine). We conducted BLASTn (National Center for Biotechnology Information, Bethesda) searches against dbEST and the non redundant set of GenBank, EMBL, and DDBJ database sequences to confirm the total gene specificity of the nucleotide sequences chosen for the primers. The specificity of the amplified PCR products was confirmed either by restriction digest or by sequencing. The 18S ribosomal RNA was measured using the Pre-Developed TaqMan Assay Reagents Endogenous control kit from Applied Biosystems (Foster City, CA).

TABLE I—SEQUENCE OF PRIMERS AND TaqMan PROBES USED FOR RT-PCR STUDIES

Gene and accession number	Position	Sequence	Size	Cycles
MMP-2 FP	1740F	5'-AGATCTTCTTCTTCAAGGACCGTT-3'	225 bp	33
MMP-2-RP NM_004530	1964R	5'-GGCTGGTCACTGGCTTGGGGTA-3'		
MMP-9-FP	1592F	5'-GCGGAGATTGGGAACCACTGTA-3'	208 bp	37
MMP-9-RP J05070	1800R	5'-GACGCGCTGTGTACACCCACA-3'		
MMP-14-FP	1288F	5'-GGATACCCAATGCCCATTTGGCCA-3'	221 bp	32
MMP-14-RP NM_004995	1508R	5'-CCATTGGGCATCCAGAAGAGAGC-3'		
TIMP1-FP	78F	5'-CATCCTGTTGTTGCTGTGGCTGAT-3'	168 bp	33
TIMP1-RP M12670	245R	5'-GTCATCTTGATCTCATAACGCTGG-3'		
TIMP-2-FP	78F	5'-CTCGCTGGACGTTGGAGGAAAGAA-3'	155 bp	30
TIMP-2-RP NM_003255	245R	5'-AGCCCATCTGGTACCTGTGGTTCA-3'		
VEGF-FP	1208F	5'-CCTGGTGGACATCTTCCAGGAGTA-3'	479 bp 407 bp 347 bp 275 bp	33
VEGF-RP AH001553	1687R	5'-CTCACCCTCGGCTGTGCACA-3'		
28S rRNA-RP	12403F	5'-GTTACCCCACTAATAGGGAACGTGA-3'		
28S rRNA-RP U13369	12614R	5'-GATTCTGACTTAGAGGCGTTCACT-3'		
VEGFR1-FP	2438F	5'-TCCCTTATGATGCCAGCAAGT-3'	79 bp	40
VEGFR1-RP	2516R	5'-CCAAAAGCCCCTCTTCAA-3'		
VEGFR1 Probe AF063657	2469	5'-CCGGGAGAGACTTAACTGGGCAAATCA-3'		
sVEGFR1-FP	2209F	5'-ACAATCAGAGGTGAGCACTGCAA-3'	180 bp	40
sVEGFR1-RP	2388R	5'-TCCGAGCCTGAAAGTTAGCAA-3'		
sVEGFR1 Probe U01134	2257	5'-TCCAAATTTAAAGCACAAAGGAATGATTGTACCAC-3'		
VEGFR2-FP	791F	5'-CTTCGAAGCATCAGCATAAGAACT-3'	156 bp	40
VEGFR2-RP	946R	5'-TGGTCATCAGCCCACTGGAT-3'		
VEGFR2 Probe AF063658	820	5'-AACCGAGACCTAAAAACCCAGTCTGGGAGT-3'		
NRPI-FP	1831F	5'-CACAGTGGAAACAGGTGATGACTTC-3'	112 bp	40
NRPI-RP	1942R	5'-AACCATATGTTGGAACTCTGATTGT-3'		
NRPI Probe XM_034725	1883	5'-CCACAGAAAAGCCACGGTCATAGACA-3'		

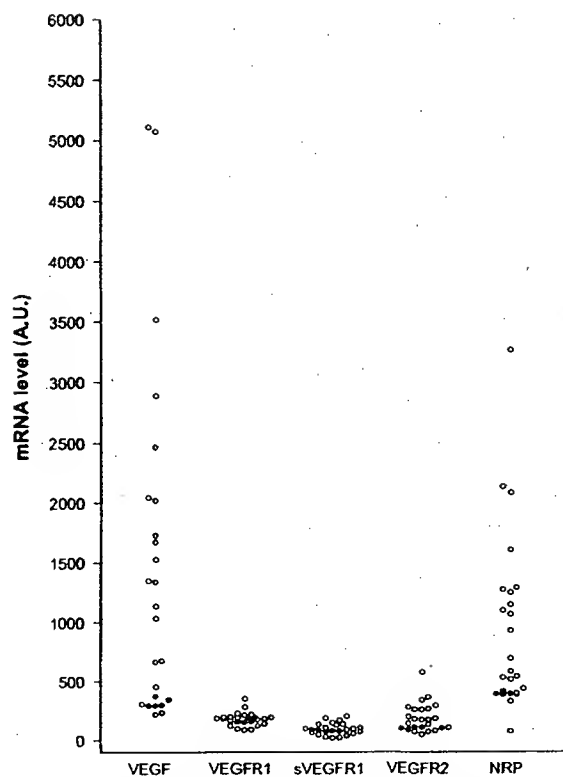


FIGURE 1 – VEGF and VEGF receptors mRNA quantification: scatter of the distribution. Normal brain (black spots) and GBMs (white spots) mRNA levels are expressed as normalized values (as described in Material and Methods: A.U. = arbitrary units). Each point represents the mean of 3 separate experiments.

*End point quantitative PCR for MT1-MMP, MMP-2, MMP-9, TIMP-2 mRNA and VEGF mRNA isoforms*

MT1-MMP, MMP-2, MMP-9, TIMP-2 and VEGF mRNA isoforms (VEGF<sub>189</sub>, VEGF<sub>165</sub>, VEGF<sub>145</sub> and VEGF<sub>121</sub>) were measured in 10 ng aliquots of cDNA using Taq polymerase (Takara, Shiga, Japan) and 5 pmol of each primers (Table 1). The thermal cycling conditions included 2 min at 95°C for denaturation and then amplification 15 sec at 94°C, 20 sec at 66°C and 20 sec at 72°C (30 sec for VEGF isoforms) with a final incubation 2 min at 72°C. PCR products were resolved on 2% Nusieve 3:1 agarose gels (BioWhittaker, Rockland, MD) and analyzed using a Fluor-S Multimager (Bio-Rad, Hercules, CA) after ethidium bromide staining. Specific mRNA levels were expressed as the ratio of specific transcripts/28S transcripts. Experiments were repeated at least 3 times in duplicate.

*Real-time quantitative PCR for VEGFR-1, sVEGFR-1, VEGFR-2 and NRPI mRNA*

Real-time quantitative RT-PCR analyses for VEGFR-1, sVEGFR-1, VEGFR-2, NRPI mRNAs and 18S rRNA were carried out using the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems). The sequences of the PCR primer pairs and fluorogenic probes that were used for each gene are shown in Table 1. A standard curve was generated by 5-fold serial dilution of placenta cDNA to cover the range of 50,000–80 ng and was run in duplicate during every experiment. For each experimental sample, the amount of target gene was determined from this standard curve. The relative expression level of the target gene was normalized against 18S rRNA to compen-

sate for variation in the quality of RNA and the amount of input cDNA (as described by the manufacturer PE Applied Biosystems in User Bulletin 2). PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 5 µl of diluted cDNA (equivalent to 10 ng total RNA), 200 nM of the probe, and 400 nM primers in a 25 µl final reaction mixture. After a 2 min incubation at 50°C to allow for UNG cleavage, AmpliTaq Gold was activated by an incubation for 10 min at 95°C. Each of the 40 PCR cycles consisted of 15 sec of denaturation at 95°C and hybridization of probe and primers for 1 min at 60°C.

To confirm amplification specificity, the PCR products were also examined by subsequent 2% agarose gel electrophoresis. Experiments were repeated at least 3 times in duplicate.

*Immunohistochemistry for VEGF and MT1-MMP*

Sections (4 µm thick) were cut from formalin-fixed, paraffin embedded tumour tissue. They were hydrated through graded alcohols and incubated in H<sub>2</sub>O<sub>2</sub> (0.3% 15 min). Sections were autoclaved for 11 min at 126°C in citrate buffer pH6 for antigen retrieval (Dako, Glostrup, Denmark). For double immunostaining sections were incubated in primary monoclonal Ab anti-MT1-MMP (Ab-4) 1:100 (Oncogene Research Products, San Diego, CA) followed by peroxidase-conjugated EnVision (Dako). Immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB+, Dako). Sections were then incubated with polyclonal Ab anti-VEGF 1:150 (Santa Cruz, Santa Cruz, CA) for 1 hr at room temperature, followed by alkaline phosphatase-conjugated EnVision (Dako). Immunoreactivity for VEGF was visualized with Fast Red chromogenic substrate (Dako). Single immunostaining was also carried out on serial sections using each primary antibody alone with the corresponding enzyme-chromogene combination. Negative controls were obtained by omitting the primary antibodies.

*Gelatin zymography assay*

MMP-2 and MMP-9 activities were quantified by gelatin zymography on 2 normal brains and 18 GBMs. Ten cryosections (10 µm) were homogenized in buffer (0.1 M Tris-HCl pH 8.1, 0.4% Triton X-100) and centrifuged for 20 min at 5,000g. The pellets were discarded. 25 µg of total protein from homogenate supernatants were mixed with non reducing sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.1% bromophenol blue) and electrophoresed directly on 10% SDS-polyacrylamide gels (SDS-PAGE) containing 0.1% gelatin (w/v).<sup>32</sup> After electrophoresis, gels were washed for 1 hr at room temperature in a 2% (v/v) Triton X-100 solution to remove SDS, transferred to a buffer (50 mM Tris-HCl, pH 7.6, containing 10 mM CaCl<sub>2</sub>) and incubated for 18 hr at 37°C. Gels were stained for 30 min with 0.1% (w/v) Coomassie brilliant blue G250 in 45% (v/v) methanol/10% (v/v) acetic acid and destained in 10% (v/v) acetic acid/20% (v/v) methanol. Gels were analyzed with Quantity One software (version 4.2.2, Bio-Rad Laboratories, Hercules, CA) after densitometric scanning of the gels using a Fluor-S Multimager (BioRad).

*Western blot*

MT1-MMP protein levels were analyzed in 2 normal brains and 15 GBMs. Brain extracts (25 µg) were mixed with 1/2 sample buffer [0.25 M Tris (pH 6.8), 10% SDS (w/v), 4% sucrose (v/v), 5% β-mercaptoethanol (v/v) and 0.125% bromophenol blue (w/v)] and boiled for 5 min. They were separated on 10% SDS-PAGE gels and transferred to a PVDF filter (NEN, Boston, MA). After blocking with 5% milk (w/v), 0.1% tween 20 (w/v) in PBS for 2 hr at room temperature, membranes were exposed to the primary antibody (10 µg/ml, clone 113-5B7, Ab-4, Oncogene Research Products, San Diego, CA) at 4°C overnight followed by incubation with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (1.3 µg/ml, Dako, Glostrup, Denmark). Signals were detected with an enhanced chemoluminescence (ECL) kit (NEN, Boston, MA). The relative intensities of the immunoreactive bands were analyzed with Quantity One software (version 4.2.2, Bio-Rad).

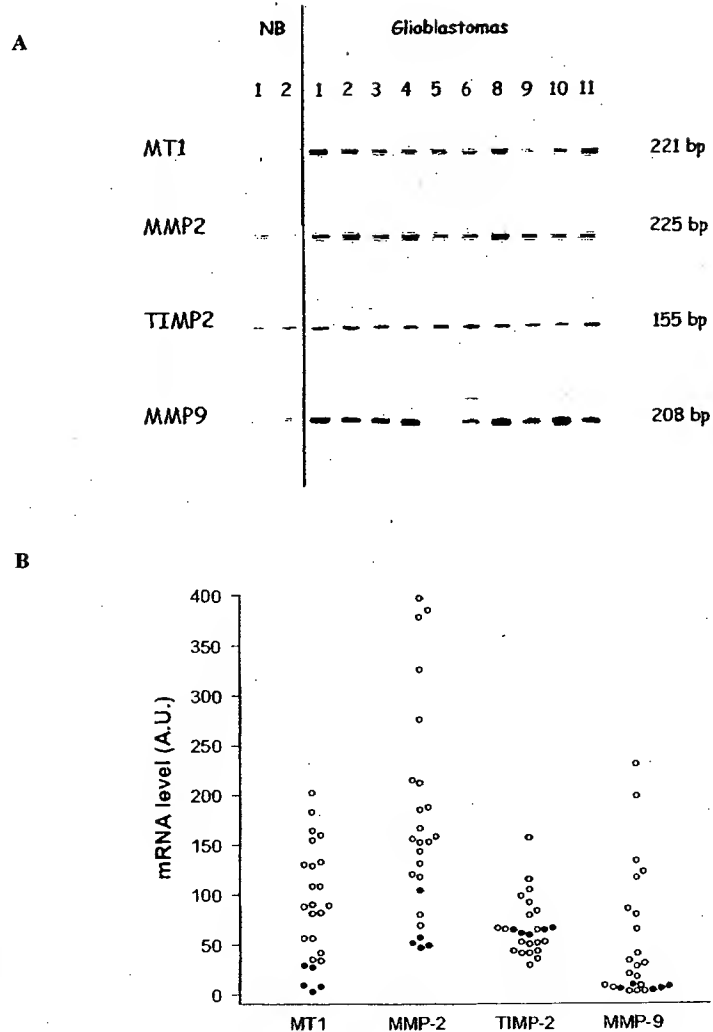


FIGURE 2—MMPs and TIMP-2 mRNA quantification. (a) Representative 2% agarose gels of RT-PCR products for MT1-MMP, MMP-2, TIMP-2 and MMP-9 in 2 normal brains (NB) and 10 GBMs. (b) Scatter plots (as described in Fig. 1). Experiment was repeated at least 3 times in duplicate.

Laboratories) after densitometric scanning of the X-ray films using a Fluor-S Multimager (Bio-Rad).

#### Statistics

VEGF, VEGFRs, MMPs and TIMP-2 expression values in GBMs were correlated using Spearman's test. Correlation was considered significant for 2-tailed  $p$ -value  $< 0.05$ . Statistical analysis was carried out using the Prism 3.0 software (GraphPad, San Diego, CA).

#### RESULTS

##### Expression of VEGF and VEGF receptors

VEGF mRNA was present in normal brains (295–375, arbitrary units; mean = 322) and in all GBM samples (217–5,112; mean = 1,774) as reported previously (Fig. 1).<sup>31</sup> In most GBMs, VEGF mRNA levels were raised 2–15-fold above normal brain values. The most abundant isoform in all cases was VEGF<sub>165</sub>, followed by VEGF<sub>121</sub>, VEGF<sub>189</sub> and VEGF<sub>145</sub> (data not shown). VEGFR-1 expression was found at similar levels in GBMs (89–357; mean = 182) and normal controls (154–198; mean = 181). There was no correlation between VEGFR-1 and VEGF mRNA levels ( $p = 0.35$ ) in GBMs. VEGFR-2 was expressed in all GBMs (48–582;

mean = 210) and in 8/20 cases at least twice normal values (87–111; mean = 103). VEGF and VEGFR-2 expressions were correlated significantly ( $p = 0.0035$ ) in GBMs. NRP1 expression varied broadly between GBMs (75–3,260; mean = 1,061) contrasting with a constant baseline expression in normal controls (383–397; mean = 390). In tumours, NRP1 correlated with VEGFR-2 ( $p = 0.0119$ ) but not with VEGF ( $p = 0.084$ ), nor VEGFR-1 ( $p = 0.066$ ). sVEGFR-1 was expressed at low levels both in normal brains (84–92; mean = 87) and GBMs (25–208; mean = 101). sVEGFR-1, however, was found to correlate with VEGFR-1 ( $p = 0.0289$ ), VEGFR-2 ( $p = 0.0029$ ), and NRP1 ( $p = 0.0027$ ) but not with VEGF ( $p = 0.053$ ).

##### Expression of MMPs and TIMP-2

MT1-MMP, MMP-2 and MMP-9 were expressed in both normal brains and GBMs but at much higher levels in the latter (Fig. 2a). MT1-MMP mRNA levels were constantly higher in GBMs (34–202; mean = 106) than in normal controls (3–29; mean = 15). MMP-2 and MMP-9 mRNA levels were higher than controls in 18/20 and 14/20 cases respectively (Fig. 2b) and correlated with each other ( $p = 0.0187$ ). MT1-MMP mRNA levels correlated with MMP-2 ( $p = 0.0008$ ) and MMP-9 ( $p = 0.005$ ). TIMP-2 had a non-discriminative distribution in relation to the controls. TIMP-2

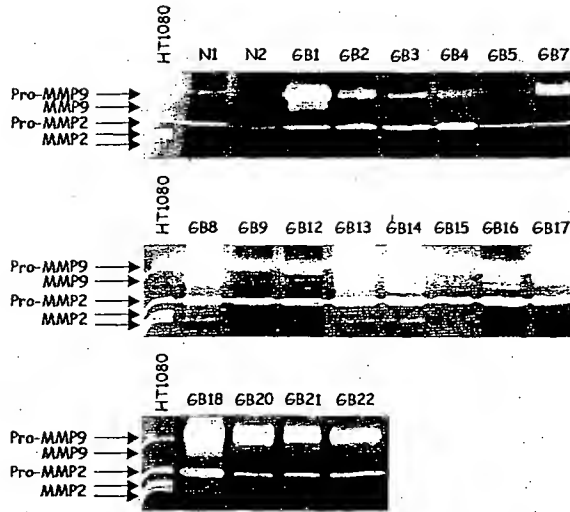


FIGURE 3—Zymographic analysis of MMP-2 and MMP-9 in tissue extracts either from normal brain (N) or GBMs (GB). Medium conditioned by human HT1080 cells was included as positive control. Positions of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 are indicated by arrows.

TABLE II—ZYMOGRAMS AND WESTERN BLOT QUANTIFICATION OF MMPs IN NORMAL BRAIN AND GBMs<sup>1</sup>

	Gelatin zymography				Western blot
	proMMP-9	MMP-9	proMMP-2	MMP-2	MT1-MMP
N1	0.41	0.00	1.52	0.00	0.44
N2	0.28	0.00	0.83	0.00	0.47
GB1	9.00	1.25	2.45	0.35	1.58
GB2	4.28	0.00	2.88	0.09	0.47
GB3	1.67	0.00	2.86	0.00	0
GB4	1.40	0.00	4.33	0.03	0
GB5	0.47	0.00	1.15	0.00	ND
GB7	5.29	0.00	1.12	0.00	0
GB8	5.75	3.78	4.63	0.65	0.55
GB9	5.02	0.00	4.09	0.00	0.45
GB12	5.08	0.00	4.79	0.20	3.18
GB13	6.86	4.58	4.93	0.48	1.55
GB14	5.59	6.75	5.53	0.76	0.94
GB15	8.00	0.00	5.13	0.00	ND
GB16	5.55	1.10	5.66	0.00	2.50
GB17	6.61	2.04	6.00	0.00	0
GB18	14.79	4.34	8.20	0.40	1.97
GB20	18.85	0.00	2.12	0.00	0
GB21	16.52	0.00	2.37	0.00	ND
GB22	20.66	0.00	5.11	0.00	0.86

<sup>1</sup>Extracts expressed as arbitrary units. N, normal brain; GB, glioblastoma; ND, not determined.

was correlated with MT1-MMP ( $p = 0.0019$ ) and MMP-2 ( $p = 0.0002$ ) but not with MMP-9 ( $p = 0.1408$ ).

#### Correlation between MT1-MMP protein and activated MMP-2 and -MMP-9

By gelatin zymography, pro-MMP-2 and pro-MMP-9 were detected in the 18 GBMs and 2 controls examined (Fig. 3, Table II). In most GBMs, levels of these inactive forms were higher than in normal brains and were correlated with their respective mRNA levels (MMP-2:  $p < 0.0001$ ; MMP-9:  $p = 0.01$ ). Activated forms of MMP-2 and MMP-9 were not found in normal brain. By contrast, they were present in 8/18 (MMP-2) and 7/18 (MMP-9) GBMs. MT1-MMP protein levels were quantified by Western blot in 15 GBMs and 2 normal brains (Fig. 4, Table II). They were signifi-

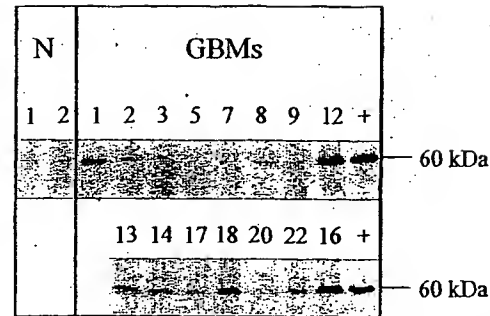


FIGURE 4—Western blot analysis using the ab4 antibody (clone 113-5B7) raised against the catalytic domain of MT1-MMP. Protein extracts from MT1-MMP transfected A2058 cells (clone SL5,<sup>28</sup>) were used as a positive control (+). MT1-MMP protein is detected in normal brain (N) and GBMs.

TABLE III—CORRELATION BETWEEN VEGF, VEGF RECEPTORS, MMPs AND TIMP-2 EXPRESSED AS  $p$  VALUES DERIVED FROM SPEARMAN'S TEST

	MT1-MMP	MMP-2	MMP-9	TIMP-2
VEGF	0.0250	0.0245	0.0053	0.0094
VEGFR-1	0.0073	0.0710	<0.0001	0.4542
VEGFR-2	<0.0001	0.0168	0.0004	0.0153
NRP1	0.0053	0.1334	0.2457	0.5480
sVEGFR-1	0.0313	0.0469	0.0194	0.2563

cantly correlated with zymogram-derived activated MMP-2 levels ( $p = 0.0226$ ) but not with activated MMP-9 levels ( $p = 0.06$ ). Interestingly, MT1-MMP protein and mRNA levels were correlated significantly ( $p = 0.089$ ), arguing for a predominantly transcriptional regulation in GBMs.

#### Correlation between VEGF network and MMPs

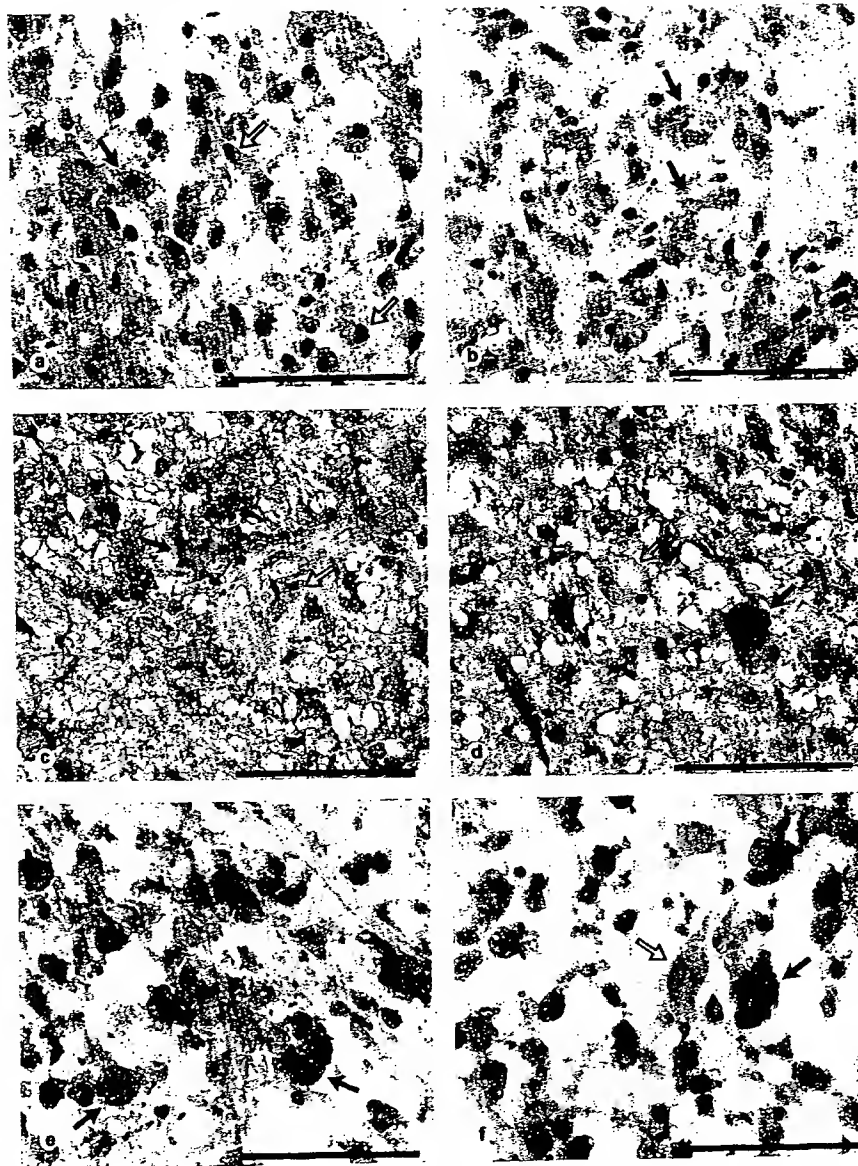
mRNA levels of VEGF and VEGF receptors were compared to MT1-MMP, MMP-2, MMP-9 and TIMP-2 (Table III). There was a significant correlation between VEGF expression and MT1-MMP, MMP-2 and MMP-9. A similar correlation was also observed between VEGFR-2 and MMPs. Interestingly, TIMP-2 expression was correlated with VEGF and VEGFR-2 but not with other VEGF receptors.

#### Immunohistochemistry for VEGF and MT1-MMP

VEGF immunoreactivity was shown in both tumour and endothelial cells, as previously reported (Fig. 5a,b).<sup>31</sup> By single immunostaining, MT1-MMP was detected in glioblastoma cells as a diffuse cytoplasmic staining (Fig. 5c,d). MT1-MMP positivity was also seen in endothelial cells and perivascular cells (Fig. 5c). By double immunostaining, we observed the co-localization of VEGF and MT1-MMP in the cytoplasm of numerous tumour cells (Fig. 5e,f).

#### DISCUSSION

GBMs are highly malignant tumours with poor prognosis. They show major microvascular proliferation and express high levels of VEGF.<sup>4-9</sup> VEGF is a strong mitogen for endothelial cells thereby promoting angiogenesis. Previous reports<sup>33,34</sup> have suggested that VEGF also stimulates tumour cell invasion, migration and survival in malignant epithelial cells through an autocrine loop by which overexpression of MMPs induces VEGF secretion and leads to subsequent amplification of cell proliferation and protection against apoptosis. We and others reported previously that in human melanoma and breast carcinoma cells, MT1-MMP upregulates VEGF expression whereas TIMP-2 reduces it.<sup>28,29,35</sup> Therefore the



**FIGURE 5** – Immunohistochemistry (scale bar = 50  $\mu$ m). (a,b) VEGF positive tumour and endothelial cells (plain arrows) show granular red staining of the cytoplasm. Negative cells (empty arrow) are seen in their close vicinity. (c) MT1-MMP positive cells show a strong brown cytoplasmic staining. They include tumour cells (plain arrow) and endothelial cells (empty arrow). (d) MT1-MMP positive tumour cells (plain arrow) are mixed with negative cells (empty arrow). (e,f) Double staining with VEGF (red) and MT1-MMP (brown). Double positive tumour cells (plain arrows) contrast with negative or single weakly positive cell (empty arrow).

pericellular proteolysis mediated by MT1-MMP in GBMs could also induce an autocrine loop resulting in enhanced VEGF expression. In turn, VEGF could act as a paracrine factor on endothelial cells to stimulate angiogenesis or possibly as an autocrine factor promoting glioblastoma cells survival-migration and invasion as demonstrated recently in the various tumour cell culture models.

We compared the expression of VEGF and its receptors with MT1-MMP, MMP-2 and MMP-9 in 20 GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels. MT1-MMP expression has been shown previously to correlate with glioma aggressiveness and its transfection in different tumour cell lines triggers an angiogenic phenotype and promotes tumour growth.<sup>20,28–30,36–38</sup> A majority of GBMs (17/20) also expressed high levels of VEGF, as previously reported, with a strong correlation between VEGF and MT1-MMP gene expression levels. Double immunostaining studies showed co-expression of VEGF and MT1-MMP by the same tumour cells. These data suggest that the transcrip-

tional control of VEGF by MT1-MMP could be operative not only *in vitro* but also *in vivo* in human GBMs.

MT1-MMP could also promote the growth of GBMs by its ability to activate MMP-2 in the presence of low concentration of TIMP-2.<sup>40</sup> Pro-MMP-2 activation occurs after the formation of a ternary complex that contains pro-MMP-2 linked to cell surface MT1-MMP via a TIMP-2 bridge. In accordance with this hypothesis, we found that MMP-2 activation occurred in 8/18 of our GBMs<sup>20,41</sup> among which 7/7 tested for MT1-MMP showed high contents of this protease.

Activated MMP-9 was also found in 7/18 of our GBMs. This is an interesting finding as active MMP-9 is able to mobilize VEGF from its ECM reservoir.<sup>39</sup> Therefore, MMPs could promote VEGF-mediated angiogenesis in GBMs by both transcriptional (MT1-MMP) and post translational (MMP-9) mechanisms.

VEGF binding to VEGFR-2 triggers the proliferation and migration of endothelial cells whereas its binding to VEGFR-1 has



opposite effects on glioblastoma cell lines.<sup>12,42</sup> In our study, VEGF mRNA levels were correlated with VEGFR-2 but not VEGFR-1, NRP1 and sVEGFR-1. Collectively our data suggest that GBMs display a specific and complex pattern of VEGF receptors, transducing VEGF signaling toward cell proliferation and migration.

In conclusion, our study adds to the evidence for an interplay between metalloproteinases and VEGF in human GBMs as previously documented in experimental tumours. Because of its dual ability to activate MMP-2 and to up-regulate VEGF, MT1-MMP might be of central importance in the growth of human glioblastomas and represent an interesting target for anti-cancer treatments.

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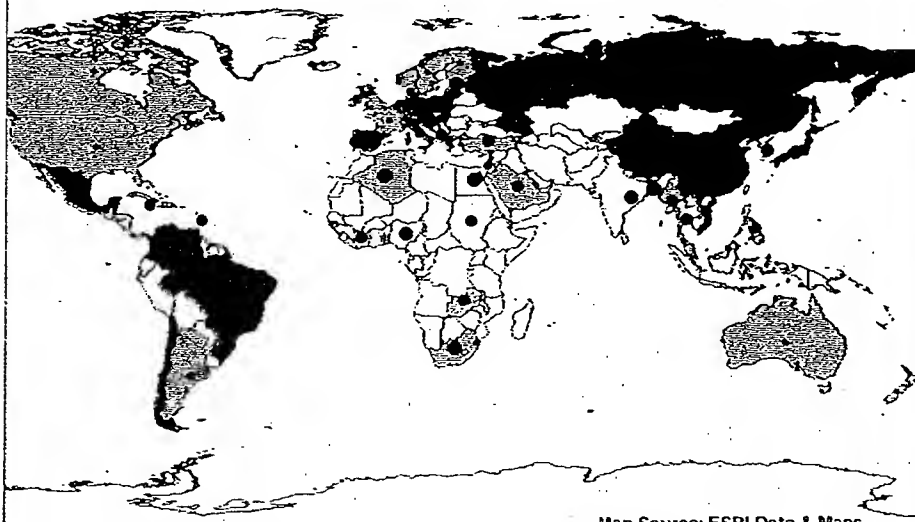
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Prevalence of *H. pylori*  
(%)

- 1st quartile (19-39.9)
- 2nd quartile (39.9-67.5)
- 3rd quartile (67.5-81.2)
- 4th quartile (81.2-96.3)

Gastric cancer incidence \*  
(100 000)

- ≤ 5
- ▨ 5-10
- 10-15
- 15-20
- > 20

\* Age and sex-standardized



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